

1 <Title page>

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3 **Gender effect on Asian neuromyelitis optica**

4 **spectrum disorder with aquaporin4-**

5 **immunoglobulin G.**

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7 Sung-Min Kim, M.D.,Ph.D.¹, Patrick Waters.,PhD. ², Mark Woodhall., Ph.D.², Yoo-Jin Kim, B.

8 A.³, Jin-Ah Kim,¹ So Young Cheon,¹ Sehoon Lee, MD⁴, Seong Rae Jo, MD, ⁴ Dong Gun Kim,

9 M.D.,⁴ Kyeong Cheon Jung, M.D. Ph.D.³ Kwang-Woo Lee. MD, PhD.¹, Jung-Joon Sung, M.

10 D., Ph.D.¹,Kyung Seok Park. MD, PhD.,^{1,4}

11

12 ¹*Dept. of Neurology, Seoul National University, College of Medicine, Seoul, Korea.*

13 ²*Nuffield Department of Clinical Neurosciences, Neuroimmunology group, Oxford, UK.*

14 ³*Dept. of Pathology, Seoul National University, College of Medicine, Seoul, Korea*

15 ⁴*Dept. of Neurology, Seoul National University, Bundang Hospital. Gyeonggi, Korea.*

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17

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Corresponding author

Kyung Seok Park, MD, PhD

Department of Neurology, Seoul National University Bundang Hospital

166 Gumi-Ro, Bundang-Gu, Seongnam-Si, Geonggi-Do, Korea

E-mail: pks1126@chol.com

Fax: 82-31-787-4059, Tel: 82-31-787-7466

Co-corresponding author

Jung-Joon Sung, M.D., Ph.D.

Department of Neurology, Seoul National University, College of Medicine

28 Yon-Gun Dong, Chong-Ro, Seoul, Korea

Tel: 82-2-2072-1015

Fax: 82-2-762-5684

E-mail: jjsaint@snu.ac.kr

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Abstract

Background: Neuromyelitis optica spectrum disorder with aquaporin4-immunoglobulin G (NMOSD-AQP4) is an inflammatory demyelinating disease characterised by a high female predominance. However, the effect of gender in patients with NMOSD-AQP4 has not been fully evaluated, particularly in Asian populations.

Objective: To determine the effect of gender in clinical manifestations and prognosis of patients with NMOSD-AQP4 among Asians.

Methods: The demographics, clinical and radiological characteristics, pattern reversal visual evoked potential (VEP) test results, and prognosis of 106 patients (18 male) with NMOSD-AQP4 were assessed.

Results: Male patients had a higher age at onset (48.7 years vs 41 years, $p = 0.037$), higher tendency to manifest as isolated myelitis (67% vs. 28%, $p = 0.005$), fewer optic neuritis attacks per year (0.08 vs. 0.27, $p < 0.001$), and shorter relative P100 latency on VEP testing (97.1% vs 108.3%, $p = 0.001$). Moreover, male gender was significantly associated with the absence of optic neuritis attacks independent of their age of onset.

Conclusion: In Asian NMOSD-AQP4 patients gender impacts on disease onset age and site of attack. This may be an important diagnostic clue in NMOSD-AQP4 patients with limited manifestations and suggests a better visual prognosis.

1 **Introduction**

2 Neuromyelitis optica spectrum disorder with aquaporin4-immunoglobulin G (NMOSD-
3 AQP4) ¹ is an inflammatory, relapsing disease of the central nervous system (CNS) that
4 is characterised by a high female predominance (>3:1).² Recent studies on patients with
5 clinical features of NMOSD ³ demonstrated that male gender was associated with
6 negative AQP4-IgG test results ⁴ and a monophasic disease course. ³ However, among a
7 homogeneous group of patients with NMOSD-AQP4, the effects of gender on the
8 clinical manifestations, severity, and prognosis of the disease remain unclear.

10 **Materials and methods**

11 *Patients and diagnostic evaluation*

12 525 consecutive patients who were suspected of having inflammatory demyelinating
13 disease of the central nervous system (IDD) were screened. They were diagnosed with
14 multiple sclerosis (n = 58) ⁶, definite NMO (n = 53) ⁷ or other clinical features of
15 NMOSD (n = 222) ¹, acute disseminated encephalomyelitis (n = 9) ⁸, or other IDD (n =
16 183) as acute transverse myelitis ⁹, optic neuritis (ON) ¹⁰, or clinically isolated
17 syndrome of the brain ¹¹; all had visited the Seoul National University Hospital or Seoul
18 National University Bundang Hospital between 1 November 2010 and 30 November
19 2014 and were followed more than a year. Eighty-one patients who had neurological
20 diseases other than IDD (19 tumor, 11 vascular disease, 7 encephalitis, 7 peripheral

neuropathy, 6 psychogenic syndrome, 5 degenerative spinal disease, 4 ocular disease, 4 hereditary disease, 4 metabolic disease, 3 infectious disease, 2 neurobehcet's disease, 2 increased intracranial pressure, 2 motor neuron disease, 1 leukodystrophy, 1 epilepsy, 1 syring, 1 trauma, and 1 graft versus host disease) were also included as controls to determine the cut-off value of quantitative fluorescence activated cell sorting assay (FACS) for the detection of AQP4-IgG.

AQP4 antibody assay

For all 525 consecutive patients with IDD and 81 controls, the assay for AQP4 antibody (AQP4-Ab) was performed at Seoul National University Hospital using FACS as described previously with minor modifications¹². Briefly, human embryonic kidney cells containing the SV40 large T antigen (HEK-293T) were transfected with plasmid containing Ds-Red tagged human AQP4 cDNA for the M23 isoform (Clontech) using Lipofectamine (Life Technologies, USA). After one day of culture the cells were incubated with patient serum (1:20 dilution) for 1 h at 4°C. Antibody binding was detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Plasma protein obtained by the therapeutic plasmapheresis of a seropositive NMO patient (1:30 dilution) and the serum of a healthy control were used as positive and negative controls, respectively. The same positive and negative controls were used in all assays throughout the study. The mean fluorescence intensity (MFI) values for the FITC were measured as

the binding of human IgG to the surface of live AQP4 expressing cells (Supplementary figure 1). The MFI index (MFI_i) was calculated as follows: [(MFI of patients - MFI of healthy control)/(MFI of positive control - MFI of healthy control)]. The cut-off value for the FACS-assay MFI_i was determined as 3.5 by the receiver operating characteristic curve analysis of 107 serum samples (26 from patients with definite NMO and 81 from patients without IDD). To validate the accuracy of the in-house FACS assay for AQP4-IgG, serum samples of 368 patients (287 with IDD and 81 without IDD) who agreed to undergo re-testing were also tested for AQP4-Ab by conventional cell-based assay (O-CBA) at John Radcliffe Hospital (Oxford, UK) ¹³ as an index test. (See the supplementary data for the validation process of our in-house FACS assay for AQP4-IgG).

Characteristics of patients with NMOSD-AQP4

Patients with seropositive NMOSD were dichotomised by gender. The clinical characteristics of these two groups were compared in terms of age at onset, attack frequency, lesion location, number of attacks in each CNS structure, cerebrospinal fluid (CSF) study results, Kurtzke extended disability status scale (EDSS), visual functional systems (VFS) for the EDSS score, ¹⁴ radiological severity (maximal contiguous length of the myelitis), mortality, and treatment types. Data for the CSF evaluation were used only when they were obtained within 30 days of an attack and prior to initiating steroid

pulse treatment¹⁵. The prognosis of patients were assessed in terms of time to a confirmed VFS of 4, confirmed EDSS of 6, and death, respectively.

Visual evoked potential

To investigate the association of gender, frequency of optic neuritis attack and functional impairment of the optic nerve, we retrospectively assessed the results of pattern reversal visual evoked potentials (VEP). Among all of our 106 patients, test result of VEP for 168 eyes (138 female eyes) were available. The VEP were assessed using a black and white checkboard pattern placed 100 cm from the subjects and reversed at a rate of 1.9 Hz. Measurements with a Nicolet Viking (Nicolet Biomedical, Madison, WI, USA) VEP device were standardised according to international recommendations.¹⁶ VEP stimulated by full field monocular stimulation were recorded with electrodes positioned at Oz and Fz as active and reference sites, respectively.¹⁶ The major positive peak (P100) generation failure rate and latency were measured for each tested eye.¹⁷ As both age and gender could affect the P100 latency of the VEP in healthy subjects,¹⁸ we also calculated the relative P100 latency to adjust the effect of age and gender on the P100 latency, by the following equation; relative P100 latency = (P100 latency / estimated normative value of P100 latency for age and gender). The estimated normative value of the P100 latency for age and gender was obtained according to the results of the previous study.¹⁸

1 *Standard protocol approval, registration, and patient consent*

2 This study was approved by the institutional review boards of Seoul National University
3 Hospital (IRB number: H-1012-023-317) and Seoul National University Bundang
4 Hospital (IRB number: B-1007-105-401). All patients provided written informed
5 consent prior to participating.

7 *Statistics*

8 The Pearson *chi* square test and Student's t-test were adapted for inter-group
9 comparisons. The univariate and multivariate linear regression analyses were adapted to
10 test the association between variables. The univariate and multivariate Cox proportional
11 hazards model was used to compare time to event data. The McNemar test was
12 performed to assess the concordance of our in-house FACS assay and the index test (O-
13 CBA) for AQP4-IgG. Predictive Analytics software (ver. 18; PASW, Inc., Chicago, IL,
14 USA) was used and *P* values < 0.05 were considered statistically significant.

16 **Results**

17 *Gender-based differences in NMOSD-AQP clinical manifestations*

18 Among the 525 patients with suspected IDD, 105 (20.0 %) tested positive for AQP4-
19 IgG by FACS assay. Moreover, five additional patients tested positive only by O-CBA
20 (supplementary data). Of those 110 patients, eight with incomplete medical records or a

1 follow-up period < 1 year were excluded. Therefore, a total of 102 patients (18 male,
2 17.6%) were finally included. When we compared the clinical manifestations by gender,
3 a higher proportion of male patients manifested as isolated myelitis (67% vs 28%,
4 respectively; $p = 0.005$; Figure 1), they experienced less ON at disease onset, had less
5 frequent ON attacks and had an older mean age at onset. There was no difference
6 between the groups in the CSF, EDSS at first attack, number of patients with a maximal
7 EDSS ≥ 6 at first attack, confirmed VFS ≥ 4 , mortality rate, or maximal spinal cord
8 lesion length, although the proportion of patients with confirmed EDSS ≥ 6 was higher
9 in males, it did not reach statistical significance (Table 1).

11 *Outcomes*

12 The Cox proportional hazards model showed that the male gender was predictive of
13 reaching 'confirmed EDSS of 6' outcomes [Figure 3A; HR 3.3; 95% confidence interval
14 1.1-10.2; $p = 0.035$]. As the male group had older age of onset (Table 1) which could be
15 a predictor of poor motor outcomes ¹⁹, both the 'male gender' and 'age of onset' were
16 tested as dependent variables in multivariate cox regression analysis for the
17 multivariable cox regression analysis with the time to 'confirmed EDSS of 6'. Multiple
18 stepwise analyses were conducted to optimize the joint effect of the variables.
19 Multivariate cox regression analysis revealed that only the age of onset, but not the male
20 gender, was significantly predictive of reaching 'confirmed EDSS of 6' outcomes (HR

1 1.07 for each year of increasing age at onset; CI 1.02–1.11; $p = 0.004$). The Cox
2 proportional hazards model did not show significant difference between two groups in
3 terms of time to VFS of 4 or death. (Figure 3B and C).

4 5 *Gender effect on the presence of optic neuritis or myelitis during follow up*

6 We assessed the association of the gender with the presence of optic neuritis or myelitis
7 during follow up. As our male patients had higher age of onset than females, both the
8 male gender and age of onset were tested as dependent variables using multivariate
9 stepwise regression analysis for either the presence of optic neuritis or myelitis,
10 independently. Our multivariate regression analysis revealed that that the presence of
11 optic neuritis was only significantly associated with the female gender (HR 0.23; CI
12 0.047-0.554, $p=0.021$), however presence of myelitis is rather associated with the age of
13 onset (HR 0.205; CI 0.000-0.011; $p=0.040$) but not with the gender (Table 2).

14 15 *Gender effect on the VEP test results in NMOSD-AQP patients*

16 To evaluate whether the more frequent optic neuritis attacks in female group actually
17 caused a poorer visual outcome, we assessed the test results of VEP. In the VEP testing
18 the male group had a significantly shorter P100 latency (104.7 ms \pm 12.0 vs. 113.3 ms \pm
19 21.5, respectively; $p = 0.014$). Moreover, they also had a significantly shorter relative
20 P100 latency (97.1 % \pm 10.5 vs. 108.3 % \pm 20.9, respectively; $p = 0.001$), which

1 implied that this inter-group difference in the P100 latency was independent of the
2 physiologic effect of age or gender. (Figure 2) The male group did not differ from the
3 female group in terms of P100 generation failure rate or time from the disease onset to
4 VEP testing (Table 1).

6 **Discussion**

7 The present study suggests that male gender can affect the site of attack and age of
8 disease onset among Asian patients with NMOSD-AQP4. Among our 102 patients with
9 NMOSD-AQP4, 18 male patients had higher age of onset, less frequent ON attacks,
10 more common manifestations of isolated myelitis, and shorter P100 latency on VEP
11 testing than females.

12 Several previous studies addressed the gender effect, among patients with clinical
13 features of NMOSD that included AQP4-Ab negative cases, and revealed that female
14 gender was associated with a relapsing disease course⁵ and AQP4-Ab positivity.⁴
15 However, since the pathogenesis and/or clinical phenotype of NMOSD without AQP4-
16 Ab can be distinct from NMOSD with AQP4-Ab,²⁰ it remains important to identify the
17 effect of gender among a homogenous group of AQP4-Ab positive cases.

18 A previous study on the prognostic factors in NMOSD-AQP4 patients in diverse
19 populations including patients from the UK and Japan reported worse visual outcomes
20 in males than females, which is in contrary to our results.¹⁹ The different study designs

1 may impact on the results as the UK/Japan study examined the visual deficit in the best
2 eye whereas we tested eyes individually¹⁹. There were also differences in ethnic
3 background as only a single Asian male patient was included in their study.^{21,22} We
4 have also considered the possibility that this discrepancy could have stemmed from
5 differences in test accuracies of our AQP4-IgG assay and the assay used in the previous
6 study performed at the University of Oxford. However, our in-house assay showed a
7 high concordance rate to those performed at the University of Oxford (supplementary
8 data). Therefore, the accuracy of the AQP4-IgG assays among research institutes does
9 not seem to be responsible for this discrepancy.

10 The majority (67%) of the male patients in this study presented as clinical forms of
11 isolated myelitis (mostly as recurrent forms, Figure 1A). They had fewer ON relapses
12 and a better VEP outcome as a result. The exact reason that male gender can affect the
13 site of attack among Asian NMOSD-AQP4 patient is still unclear. One hypothesis is the
14 difference in the expression of gender hormone receptor subfamilies (specifically,
15 oestrogen receptors α and β , which are involved in inflammation²³ and demyelination²⁴,
16 respectively) in the diverse types of CNS tissues.²⁵ However, further studies with
17 experimental models are needed to identify the exact cause of our finding, which might
18 be a useful clue in identifying beneficial environmental or hormonal factors in
19 NMOSD-AQP4.

1 The male patients had a higher likelihood of motor disability than females in our study
2 which may be due to the higher age of onset in male group rather than the male gender
3 itself (Figure 3A). This finding replicates several previous studies that reported a poor
4 motor outcomes in elderly-onset NMOSD-AQP4 patients.^{19, 26}
5 Multi-centre prospective studies with more detailed ophthalmologic examinations and a
6 greater number of male patients (n = 18 in our study) may shed further light on our data.
7 In addition our analysis included all eyes to evaluate visual outcome and avoid missing
8 subclinical optic neuritis. However, examination of only eyes with ON would allow a
9 comparison of severity of symptomatic optic neuritis in each group.
10 Nevertheless, we demonstrate that male gender can affect the site of attack and age of
11 onset in Asian patients NMOSD-AQP4, as our male patients had less optic neuritis with
12 better VEP test results, more isolated myelitis, and had an older age of onset than female
13 patients. We speculate that our finding will be an important clue in identifying NMOSD-
14 AQP4 patients with limited manifestations as well as in predicting their clinical courses.

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7 The Authors declare that there is no conflict of interest. PW and the University of
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Table 1. Characteristics of NMOSD-AQP4 by gender

	Total	Male	Female	<i>P</i> <i>value</i>
Number	102	18	84	
Age at onset , mean \pm SD (min - max), y	42.3 \pm 14.2 (9.8 - 75.3)	48.7 \pm 15.3 (18.2 - 72.8)	41 \pm 13.7 (9.8 - 75.3)	0.037
Follow-up duration , mean \pm SD (min - max), y	7.37 \pm 7.33 (1.01 - 31.83)	7.45 \pm 8.32 (1.21 - 31.33)	7.35 \pm 7.16 (1.01 - 31.83)	<i>n.s.</i>
Cumulative number of attacks , mean \pm SD (min - max)				
During follow-up period	3.1 \pm 2 (0 - 14)	2.8 \pm 2 (0 - 7)	3.2 \pm 2 (1 - 14)	<i>n.s.</i>
During 1 year of disease onset	1.5 \pm 0.8 (0 - 4)	1.4 \pm 0.7 (0 - 3)	1.6 \pm 0.9 (0 - 4)	<i>n.s.</i>
Mean annual relapse rate/year	0.69 \pm 0.53 (0 - 3.25)	0.6 \pm 0.51 (0 - 2.05)	0.7 \pm 0.54 (0.1 - 3.25)	<i>n.s.</i>
First attack location, number of patients (%)				
Optic nerve	40 (39.2)	3 (16.7)	37 (44.1)	0.026
Spinal cord	53 (52.0)	14 (77.8)	39 (46.4)	0.014
Brain	17 (16.7)	1 (5.6)	16 (19.1)	<i>n.s.</i>
Frequency of attacks in each structures , mean \pm SD (min - max)				
Optic nerve/year	0.24 \pm 0.37 (0 - 2.44)	0.08 \pm 0.19 (0 - 0.74)	0.27 \pm 0.39 (0 - 2.44)	0.00
Spinal cord/year	0.44 \pm 0.46 (0 - 2.24)	0.53 \pm 0.55 (0 - 2.05)	0.42 \pm 0.44 (0 - 2.24)	<i>n.s.</i>
Brain, year	0.08 \pm 0.2 (0 - 1.41)	0.03 \pm 0.09 (0 - 0.31)	0.09 \pm 0.22 (0 - 1.41)	<i>n.s.</i>
Pattern reversal visual evoked potential				
Number of tested eyes	162	28	134	
Presence of optic neuritis before VEP testing	79 (48.7%)	7 (25%)	72 (53.7%)	0.007
P100 generation failure (%)	41 (25.3%)	7 (25%)	34 (25.4%)	<i>n.s.</i>
Age at VEP testing	49.5 \pm 12.8 (20.5 - 78.7)	55.8 \pm 10.8 (37.1 - 73.4)	48.3 \pm 12.9 (20.5 - 78.7)	0.006
Time from the disease onset to VEP testing (yr)	5.4 \pm 7.3 (0.01 - 29.3)	6.0 \pm 6.8 (0.28 - 21.0)	5.3 \pm 7.4 (0.01 - 29.3)	<i>n.s.</i>
Cerebrospinal fluid study , mean \pm SD (min - max)				
White blood cells (/mm ³)	8.6 \pm 11.8 (0 - 52.7)	9.8 \pm 14.7 (0 - 47)	8.3 \pm 11 (0 - 52.7)	<i>n.s.</i>
CSF/serum albumin ratio	8.6 \pm 5.7 (0 - 32)	9.6 \pm 5.9 (0 - 19.6)	8.4 \pm 5.7 (3.2 - 32)	<i>n.s.</i>
CSF/serum IgG ratio	4.7 \pm 3.6 (0 - 17.6)	5.5 \pm 4.1 (0 - 12.9)	4.5 \pm 3.4 (1.8 - 17.6)	<i>n.s.</i>
Disability and severity				
EDSS at first attack, mean \pm SD	4.1 \pm 2.6 (0 - 9.5)	3.9 \pm 3.1 (0 - 9.5)	4.1 \pm 2.5 (1 - 9.5)	<i>n.s.</i>

Maximal EDSS ≥ 6 at first attack, number (%)	20 (23.0)	4 (25.0)	16 (22.5)	<i>n.s.</i>
Confirmed EDSS ≥ 6 , number (%)	13 (13.3)	5 (27.8)	8 (10.0)	<i>0.059</i>
Confirmed visual functional score > 4, number (%)	34 (34)	3 (17.7)	31 (37.8)	<i>n.s.</i>
Death	3 (3)	1 (5.6)	2 (2.4)	<i>n.s.</i>
Maximal contiguous length of myelitis, mean \pm SD	5.9 \pm 4.5 (0 - 17)	4.4 \pm 3 (0 - 11)	6.3 \pm 4.7 (0 - 17)	<i>n.s.</i>
Types of treatment, number of patients (%)				
Oral immune suppressant*	67 (68)	10 (58.8)	60 (69.5)	<i>n.s.</i>
Rituximab	24 (24)	6 (35.3)	18 (22.0)	<i>n.s.</i>

1

2 NMOSD-AQP4, neuromyelitis optica spectrum disorders with aquaporin 4 immunoglobulin G

3

Figure 1. Patterns of clinical manifestations in each group.

Patterns of clinical manifestations by attack location were assessed in individual patients. More than half of the seropositive male patients had manifestation of isolated myelitis (mostly as recurrent form) without attacks in the optic nerve or brain.

ON, optic neuritis

Figure 2. Test results for visual evoked potentials.

Both the mean P100 latency (A) and the relative P100 latency, adjusted for age and gender, (B) were significantly shorter in male patients than females..

Figure 3. Kaplan–Meier curves to demonstrate effects of gender on the prognosis of each group.

Male patients had a higher likelihood of reaching confirmed EDSS 6 (A). However, they did not differ from females in terms of time to a VFS (B) of 4 or death (C).

EDSS, extended disability scale score; n.s., not significant; VFS, visual function score